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Original Article

Resveratrol reverse multidrug resistance in adriamycin-resistant K562/A02 cells *in vitro* and *in vivo*

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Abstract: Multidrug resistance (MDR) represents a major challenge for successful chemotherapeutic treatment of cancer. Resveratrol (RES) is believed to have multiple bioactivities including anti-cancer, prevention of cardiovascular diseases, and anti-inflammatory. This study was aimed to examine the effect of resvertrol on human leukemic MDR K562/A02 cells. Treatment of combination of resveratrol and adriamycin (ADM) resulted in potentiation of cytotoxicity detected using a cell counting kit-8 assay, and treatment with a combination of RES and ADM caused synergistic enhancement of the proliferation inhibition effect. RES increased Rh123 retention and ADM accumulation in K562/A02 cells. Furthermore, the combined treatment of RES and ADM synergistically delayed the growth of xenografts in mice. In addition, the expression of P-glycoprotein (p-gp) was significantly decreased following treatment with resveratrol alone or in combination with ADM both in vitro and in vivo. These findings demonstrated that resveratrol is important in MDR and may be developed into a new reversal agent for cancer chemotherapy.

Keywords: Resveratrol, human leukemia, multidrug resistance, adriamycin

Introduction

Chronic myelogenous leukemia (CML), a cancer of the white blood cells, is a myeloproliferative hematologic neoplasm linked to a hematopoietic stem cell disorder having an incidence 1-1.5 per 100000 inhabitants, it can lead to the increased production of granulocytes at all stages of differentiation and represent approximately 15% of all leukaemias diagnosed in adults with an onset at 40-60 years of age [1-3]. The development of multidrug resistance (MDR) is one of the predominant obstacles to the success of cancer chemotherapy, including the treatment of leukemia [4, 5]. The molecular mechanisms that lead to MDR include decreased drug accumulation in tumor cells, altered intracellular drug distribution, increased detoxification, alteration of drug targets, diminished drug-target interaction, increased DNA repair, altered cell-cycle regulation, and dysregulation of cell death pathways [6-8]. Adriamycin (ADM) is one of the most effective anticancer drugs for various types of cancers, however, the severe MDR has limited its therapeutic effectiveness [9, 10].

Resveratrol (trans-3,4',5-trihydroxystilbene, RSV) is a natural compound produced by a restricted number of plants (about 31 genera), primarily from root extracts of the oriental plant, Polygonum cuspidatum and from red grapes [11, 12]. Resveratrol is believed to have multiple bioactivities including anti-cancer, prevention of cardiovascular diseases, and antiinflammatory, and it has been identified to have a strong chemopreventive effect against the development of several cancers [13, 14]. In this study, we initially showed the reversion of multidrug resistance effect of resveratrol in K562/A02 cells. We then demonstrated the inhibitory effect of resveratrol on the apoptosis signal in K562/A02 cells. We also examined the effect of resveratrol in vivo using a model of xenografts in mice.

Materials and methods

Cell lines and cell culture

The human chronic myeloid leukemia cell lines K562, and its MDR counterpart K562/A02 were obtained from the Institute of Hematology

of Chinese Academy of Medical Sciences (Tianjin, China). K562 cells were cultured in complete RPMI-1640 at 37°C in a humidified atmosphere of 5% CO_2-95% air. K562/A02 cells were cultured in a medium with or without supplementation of 1 μg/ml adriamycin for 2 weeks prior to experiments.

Cell proliferation assay

Cell growth was analyzed using a WST-8 Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Briefly, cells were seeded into 96-well plates (5.0×10^3/well) and cultured in the presence of different drug treatment (ADM 0, 0.01, 0.1, 1, 10, 100 μM; RES 0, 4, 8, 16, 32, 64 μM) for 48 h. CCK-8 solution (10 μl) was added to each well and the cultures were incubated at 37°C for 1 h. Absorbance at 450 nm was measured using a THERMOmax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Combinational inhibition effect of RES and DOX in K562/A02 cells proliferation

5.0×10^3 K562/A02 cells per well were seeded in a 96-well plate using a medium that contained ADM (0, 2, 4, 8, 16 μM) or a combination of RES (0, 4, 8, 16 μM). Subsequent to 48 h of exposure, the cytotoxicity of the drugs was assessed via a CCK-8 assay above.

Efflux and accumulation of Rh123 was measured by flow cytometry

For determination of Rh123 efflux, cells were loaded for 60 min with Rh123 in the absence of RES, and then the medium was replaced with Rh123-free medium containing RES or the vehicle (DMSO). Following efflux intervals of 60 min, the medium was removed, and the cells were washed twice with ice-cold Hanks' balanced salt solution (HBSS) and placed in HBSS containing 10% fetal bovine serum on wet ice. The green fluorescence of Rh123 was measured using a FACS can flow cytometer. Briefly, cells (5×10^5 per sample) were incubated in the dark with 1 mg/ml Rh123 at 37°C in 5% CO_2 for 2 h. RES (8 μM, 16 μM, dissolved in 0.1% DMSO) was added to the cells at the same time as the Rh123. Following Rh123 accumulation, the cells were washed twice with ice-cold HBSS and prepared for flow cytometry.

Intracellular accumulation of adriamycin

Intracellular accumulation of Adriamycin was assessed by detecting fluorescence intensity. Briefly, K562/A02 cells were seeded into 6-well plates at a density of 1×10^6/well. Adriamycin was added to the medium to the final concentration of 10 μM in the absence or presence of RES at concentrations of 8, and 16 μM. The cells were incubated for 1 h, and then were harvested for the determination of Adriamycin accumulation. The cells were washed three times with ice-cold phosphate-buffered saline (PBS) and incubated in isopropanol overnight at -20°C. The absorbance of the supernatant was read using a fluorescence spectrofluorometer (Hitachi High-Tech Companies, Tokyo, Japan) at wavelengths of 470 and 590 nm. The value of ADM accumulation within the cells was calculated according to the standard curve.

Animal studies

Five-week-old, female BALB/C nude mice were purchased from Beijing Colab Bio-Technology Ltd., China. The animals were housed under pathogen-free conditions. The research protocol was in accordance with the institutional guidelines of the Animal Care and Use Committee at Weifang Medical University. K562/A02 cells (5×10^6) suspended in 0.1 ml of PBS were implanted subcutaneously into the right flank of nude mice to form xenograft tumors. When the tumor size reached approximately 100 mm^3, the mice were randomly divided into four groups with five mice in each group including control group, ADM group, RES group, and ADM + RES group. RES (40 mg/kg) and ADM (5 mg/kg) were administered every two days by tail intravenous injection for 14 days. The tumors and body weight of mice in each group were monitored every three days. After 14 days, the tumors were harvested.

RT-PCR analysis

Total RNA from cells and snap-frozen tumor samples was isolated using TRIzol, as recommended by the manufacturer. RT-PCR was used for the analysis of multidrugresistance gene (MDR1) and survivin mRNA with GAPDH as an internal control. Primers for MDR1 were as follows: forward primer 5'-CAAGGAAGCCAATGCCTATGAC-3', reverse primer 5'-ATCCAGAGCCACCTGAACCACT-3'. GAPDH primers were as follows: forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3'. The reactions were performed in accordance with standard protocols. PCR was performed by

Table 1. Inhibition effect of ADM K562 cells and K562/A02 cells

ADM (μM)	Inhibition rate (%)			
	K562		K562/A02	
	Mean	SD	Mean	SD
0.01	3.25	0.09	0.44*	0.10
0.1	15.67	0.69	0.88*	0.05
1	54.41	0.71	12.82*	1.39
10	77.72	2.08	31.47*	3.00
100	85.24	1.52	60.56*	1.99
IC50	0.57		10.77	

*P < 0.05, K562/A02 vs. K562.

Table 2. Inhibition effect of RES on K562 cells and K562/A02 cells

RES (μM)	Inhibition rate (%)			
	K562		K562/A02	
	Mean	SD	Mean	SD
4	1.44	0.10	0.99*	0.05
8	8.88	1.05	7.06*	0.76
16	24.82	1.61	21.82	2.39
32	41.47	2.99	35.96	2.50
64	60.56	1.99	56.11	0.54
IC50	35.9		46.1	

*P < 0.05, K562/A02 vs. K562.

an initial denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 60 s. The products were separated by electrophoresis in 2% agarose and visualized using ethidium bromide. The experiments were performed in triplicate.

Western blotting assay

Western blotting assay was performed to analyze the expressions of related protein levels in K562/A02 cells. Briefly, K562 cells (3×10^5) seeded in 6-well plates were exposed to various concentrations of resveratrol for 72 h. The cells were harvested and lysates (50 μg of protein per lane) were fractionated by 10% SDS-PAGE as described below. The proteins were electro-transferred onto PVDF membranes, and then incubated with primary antibodies overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies were added in TBST containing 5% BSA. The bound antibodies were visualized by using an enhanced chemiluminescence reagent (Millipore, USA) and quantified by densitometry using ChemiDoc XRS+ image analyzer (Bio-Rad, USA) adjusted with β-actin as loading control. Triplicate experiments with triplicate samples were performed.

Statistical analysis

All of the experiments were repeated at least three times and data were expressed as mean ± SD (standard deviation). Statistical software SPSS15.0 was used for the assessment. One-Way ANOVA was used for comparing means of multiple samples. Multiple between-group comparisons were performed using the S-N-K method. $P < 0.05$ was considered as statistically significant.

Results

RES inhibits proliferation of K562 and K562/A02 cells

Both ADM and RES were demonstrated to inhibit the growth of K562 and K562/A02cells in a dose-dependent manner. As showed in **Table 1**, the IC 50 values of ADM were 0.57 and 10.77 μM in K562 and K562/A02 cells, respectively, and there were significant differences between the inhibition rates of K562 and K562/A02 cells at the concentrations of each concentration of ADM. While the IC 50 values of RES were 35.9 and 46.1 μM in K562 and K562/A02 cells, respectively (**Table 2**). The inhibition effects of both ADM and RES are shown in **Tables 1** and **2**. Although the IC50 values of RES in K562/A02 was higher than in K562 cells, there were no significant differences demonstrated between the inhibition rates of K562 and K562/A02 cells at the concentrations of 16, 32, 64 μM of RES.

RES enhances cytotoxicity of ADM on K562/A02 cells in vitro

The reversal effect of RES on the MDR of MCF-7/DOX cells was investigated. As shown in **Table 3**, RES enhanced the inhibitory effect of DOX on cell growth in a dose-dependent manner. The IC50 values of ADM were decreased from 10.2 μM to 9, 5, 9.2 and 8.8 μM in K562/A02 cells when RES were also added at the concentration of 4, 8, 16 μM.

RES inhibited the efflux of p-gP and increased ADM accumulation within K562/A02 cells

As shown in **Figure 1A**, the fluorescence intensity of the group treated with RES was significantly higher than that of the control group, the efflux and accumulation of Rh123 in K562/A02 cells treated with 8 and 16 μM RES was signifi-

Table 3. Antitumor effect of RES Combined with ADM on K562/A02 cells indicated as inhibition rate (%)

ADM (μM)	Inhibition rate (%)							
	RES 0 μM		RES 4 μM		RES 8 μM		RES 16 μM	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
2	10.34	3.21	13.43	2.53	17.73	4.29	21.54	5.36
4	18.98	4.23	25.28	5.38	29.88	3.75	36.94	4.68
8	31.47	3.28	40.23	4.29	43.27	6.39	47.75	7.55
16	44.28	4.29	56.58	6.32	59.28	6.39	66.35	7.92
IC50	10.21		9.51		9.17		8.76	

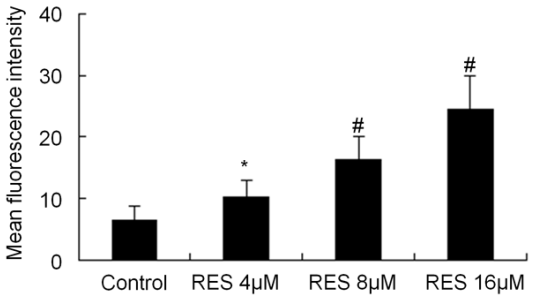

Figure 1. Effects of RES on Rh123 retention. Cells were incubated with Rh123 for 60 min, washed, and resuspended in medium with different concentrations of RES or vehicle control for 60 min. Rh123 fluorescence was measured using FACS can. The mean fluorescence intensity of RES group was significantly higher than that of the negative control group. *P < 0.05 vs. control group; #P < 0.01 vs. control group.

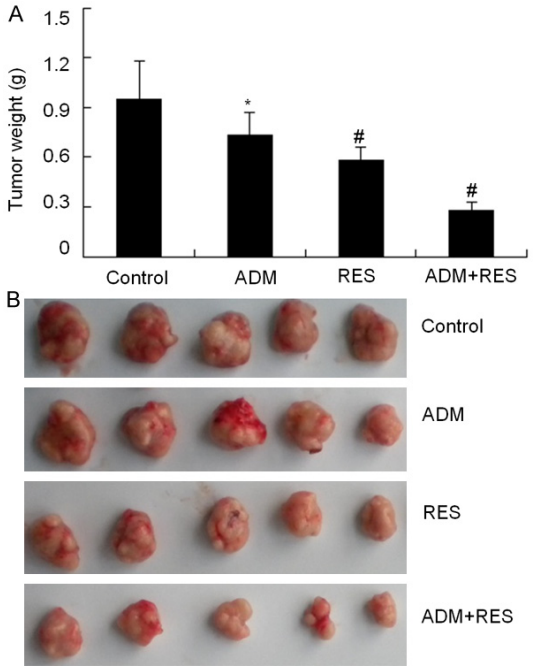

Figure 2. Effects of RES on K562/A02 tumor growth in xenograft mice model. A. Effects of RES and ADM on the tumor weight of K562/A02 tumors in nude mice; B. Tumors of different groups collected after sacrifice of mice. *P < 0.05 vs. control group; #P < 0.01 vs. control group.

cantly higher than that in control cells (P < 0.05). The results indicate that RES inhibits the efflux of P-gp, thus may increase the accumulation of drugs in MDR cells.

Then, the ADM accumulation within K562/A02 cells was detected in the presence of RES. The concentration of ADM in K562 and K562/A02 cells increased with increasing ADM treatment regardless of the RES dose, however, the concentration of ADM in K562/A02 cells was significantly lower than that in the K562 cells (P < 0.05). In addition, RES did not significantly affect the concentration of ADM in K562 cells, while it could elevate the concentration of ADM in K562/A02 cells in a dose-dependent manner.

RES decreases MDR1 gene expression and p-gP protein levels within K562/A02 cells

MDR1 mRNA expression level was detected using RT-PCR assay, and then p-gP protein level was analyzed by western blotting. In K562/A02 cells, ADM treatment did not significantly affect the expression of MDR1 mRNA and p-gp protein levels, while RES significantly suppressed the expression of MDR1 at mRNA level and decreased p-gp protein expression in K562/A02 cells both in vitro and in vivo (**Figure 2**). As shown in **Figure 2A**, the expression of p-gP protein was consistent with the RT-PCR results. And the MDR1 gene expression and p-gP protein levels in tumor tissues from mice xenografts were also consistent with the in vitro results (**Figure 2B**).

RES enhances inhibition effects of adriamycin on K562/A02 cell growth in vivo

The inhibitory effect of RES on tumor growth in vivo was evaluated in K562/A02 xenografts in nude mice. Xenografted mice were sacrificed and the tumors were collected. The tumor weight in control group was larger tumors than the other groups. The mean tumor weight of control group treated with a combination of RES and ADM (0.28±0.05 g) was much smaller than that of the control group (0.95±0.13 g),

Table 4. Effects of RES and ADM on the weight of K562/A02 tumors in nude mice and the inhibition rates of tumors (mean ± SD)

Group	Body weight (g, initial/14 days)	Tumor weight (g)	Inhibition rate (%)
Control	17.5±1.8/21.4±0.9	0.95±0.13	-
ADM	17.9±2.0/19.2±2.8*	0.73±0.14#	23.2
RES	17.7±1.5/21.6±2.3	0.58±0.08#	38.9
ADM+RES	17.8±1.6/20.9±3.2	0.28±0.05#	70.5

Established tumors were treated with ADM, RES, ADM+RES, or control normal saline. Body weight in the table was data measured before and after 14 days of drug administration. Tumors weight was obtained after the mice were sacrificed. *P < 0.05 (vs. control group body weights); #P < 0.01 (vs. Control group tumor weights).

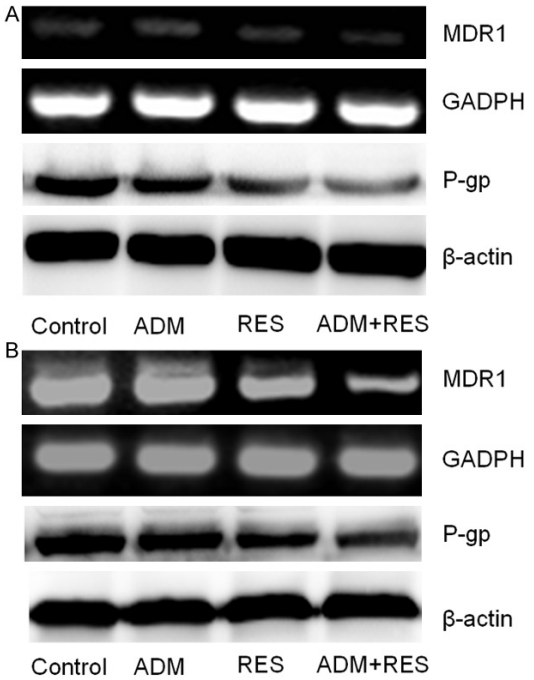

Figure 3. Inhibition of the expression of MDR1 mRNA and P-gp protein in K562/A02 cells and xenograft tumors. A. MDR1 mRNA and P-gp protein expression in K562/A02 cells. B. MDR1 mRNA and P-gp protein expression in tumor tissues of different groups. *P < 0.05 vs. control group; #P < 0.01 vs. control group.

the ADM-treated group (0.73±0.14 g), and the RES-treated group (0.58±0.08 g). As shown in **Table 4** and **Figure 3**, in the control group, ADM group, RES group, and ADM+RES group, the inhibition rates were 23.2%, 38.9%, and 70.5%, respectively (P < 0.01 vs. untreated control). Although the inhibition effects of RES and ADM combination was elevated than either of the drug used alone, there was no significant differences of the mean body weight between mice in the combination group and RES/ADM alone group.

Discussion

Resveratrol, obtained from Chinese traditional medicine, is an effective and safe agent in treatment of various diseases including cancer [11, 15, 16]. The aim of this study was to determine the drug resistance reversing effects of resveratrol both in vitro and in vivo, and to gain insight into its reversal effect and the molecular mechanism of the effects. The key findings of this study included: a) RES was able to inhibit the proliferation of K562 and K562/A02 cells in a dose-dependent manner; b) RES could reverse the MDR of the K562/A02 cells, elevate the concentration of ADM in K562/A02 cells; c) RES hindered the tumor growth of xenografts in mice model; d) RES decreased MDR1 and p-gP expression in K562/A02 cells both in vitro and in vivo.

Previous researches have proved that resveratrol could reverse multidrug esistance in human breast cancer doxorubicin-resistant cells, enhance the anti-skin cancer effects of ursolic acid [17, 18]. MDR is often regarded as the cause of the failure of chemotherapeutic drug treatments in patients diagnosed with malignancy including leukemia [19]. In this study, we found that, RES could inhibit the proliferation of K562 cells, but also significantly inhibit the proliferation of K562/A02 cells. While in the mean time, our results showed a strong resistance of ADM treatment in K562/A02 cells. Our findings revealed a strong reverse effect of RES in the MDR of the human leukemia resistant cell line K562/A02, the combination of RES and ADM resulted in a better efficacy.

The precise mechanisms of MDR are complex and have not been fully elucidated. Possible mechanisms of MDR includes extrusion of drug by cell membrane pumps, increase of drug detoxification, DNA damage repair, redistribution of intracellular drug accumulation, modification of drug target molecules, suppression of drug-induced apoptosis, up-regulation of lipids and other biochemical changes [20-22]. Although the exact mechanism remains unclear, the overexpression of drug-efflux pumps, such as P-glycoprotein (p-gp), is believed to be

a major mechanism of resistance [23, 24]. Intracellular concentrations of drugs may be affected by transporters involved in drug influx and efflux. P-gp-mediated MDR is considered to be the classic mechanism of resistance by pumping hydrophobic pro-lipid anti-cancer drugs out of cells, causing inadequate intracellular concentrations of anticaner drugs, and thus cause MDR. Our results showed that RES notably affect Rh123 retention, and increased intracellular concentration of ADM indicating that RES might have a strong reverse effect of MDR in K562/A02 cells.

P-gp is an ATP-dependent membrane transport protein encoded by the MDR1 gene, and it pumps exogenous cell substances from the inside to the outside of cells to protect tissues from exogenous toxic substances. The MDR of tumor cells can be reversed by inhibition of P-gp expression and function, which restores their sensitivity to chemotherapeutic drugs [24-26]. In this research, the expression of MDR1 mRNA and p-gP protein was consistent with the RT-PCR results, we observed a strong inhibition effect of RES in modulation of MDR1 gene expression and p-gp protein levels in K562/A02 cells both in vitro and in vivo.

In summary, resveratrol could reverse multidrug resistance in adriamycin-resistant K562/A02 cells in vitro and in vivo, the combination treatment of RES and ADM enhanced anti-proliferation effects on K562/A02 cells by modulating the expression of MDR1 gene and p-pg protein levels. Resveratrol has the potential to be developed as a promising agent for treatment of cancers with adriamycin resistant cells.

Disclosure of conflict of interest

None.

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